ANTISENSE MODULATION OF STEAROYL-COA DESATURASE EXPRESSION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of pending United States patent application No. 09/918,187, filed July 30, 2001.

BACKGROUND OF THE INVENTION

Saturated fatty acids are known to be the precursors of unsaturated fatty acids in higher organisms. However, the control mechanisms that govern the conversion of saturated fatty acids to unsaturated fatty acids are not well understood. The relative amounts of different fatty acids have effects on the physical properties of membranes. Furthermore, regulation of unsaturated fatty acids is important because they play a role in cellular activity, metabolism and nuclear events that govern gene transcription.

A critical committed step in the biosynthesis of mono-unsaturated fatty acids is the introduction of the first *cis*-double bond in the delta-9 position (between carbons 9 and 10). This oxidative reaction is catalyzed by stearoyl-CoA desaturase (SCD, also known as delta-9-desaturase) and involves cytochrome b_5, NADH (P)-cytochrome b_5 reductase and molecular oxygen (Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558). Although the insertion of the double bond occurs in several different methylene-interrupted fatty acyl-CoA substrates, the preferred substrates are palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA respectively (Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558).

It has been recognized that, regardless of diet, the major storage fatty acids in human adipose tissue are oleic acid, an 18 carbon unsaturated fatty acid, and palmitoleic acid, a 16 carbon unsaturated fatty acid (Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558). During the *de novo* synthesis of fatty acids, the fatty acid synthase enzyme stops at

palmitoleic acid but the end product of the pathway is usually oleic acid (Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558).

The stearoyl-CoA desaturase gene was partially characterized in 1994 via isolation of a 0.76 kb partial cDNA from human adipose tissue (Li *et al.*, *Int. J. Cancer*, **1994**, *57*, 348-352). Increased levels of stearoyl-CoA desaturase mRNA were found in colonic and esophageal carcinomas and in hepatocellular carcinoma (Li *et al.*, *Int. J. Cancer*, **1994**, *57*, 348-352). The gene was fully characterized in 1999 and it was found that alternative usage of polyadenylation sites generates two transcripts of 3.9 and 5.2 kb (Zhang *et al.*, *Biochem. J.*, **1999**, *340*, 255-264). Two loci for the stearoyl-CoA desaturase gene were mapped to chromosomes 10 and 17 and it was determined that the chromosome 17 loci encodes a transcriptionally inactive pseudogene (Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558).

A nucleic acid molecule encoding the human stearoyl-CoA desaturase and a nucleic acid molecule, which under suitable conditions, specifically hybridizes to the nucleic acid molecule encoding the human stearoyl-CoA desaturase, have been described (Stenn *et al.*, International patent publication WO 00/09754, 2000).

Stearoyl-CoA desaturase affects the ratio of stearate to oleate, which in turn, affects cell membrane fluidity. Alterations of this ratio have been implicated in various disease states including cardiovascular disease, obesity, non-insulin-dependent diabetes mellitus, skin disease, hypertension, neurological diseases, immune disorders and cancer (Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558). Stearoyl-CoA desaturase has been viewed as a lipogenic enzyme not only for its key role in the biosynthesis of monounsaturated fatty acids, but also for its pattern of regulation by diet and insulin (Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558).

The regulation of stearoyl-CoA desaturase is therefore of considerable physiologic importance and its activity is

sensitive to dietary changes, hormonal imbalance, developmental processes, temperature changes, metals, alcohol, peroxisomal proliferators and phenolic compounds (Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558).

Animal models have been very useful in investigations of the regulation of stearoyl-CoA by polyunsaturated fatty acids (PUFAs). For example, in adipose tissue of lean and obese Zucker rats, a 75% decrease in stearoyl-CoA desaturase mRNA was observed when both groups were fed a diet high in PUFAs relative to a control diet (*Jones et al.*, *Am. J. Physiol.*, **1996**, *271*, E44-49).

Similar results have been obtained with tissue culture systems. In the murine 3T3-L1 adipocyte cell line, arachidonic linoleic, linolenic, and eicosapentanenoic acids decreased stearoyl-CoA desaturase expression in a dose-dependent manner (Sessler *et al.*, *J. Biol. Chem.*, **1996**, *271*, 29854-29858).

The molecular mechanisms by which PUFAs regulate stearoyl-CoA desaturase gene expression in different tissues are still poorly understood. The current understanding of the regulatory mechanism involves binding of PUFAs to a putative PUFA-binding protein, after which repression transcription occurs via binding of the PUFA-binding protein to a *cis*-acting PUFA response element of the stearoyl-CoA desaturase gene (SREBP) (Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558; Zhang *et al.*, *Biochem. J.*, **2001**, *357*, 183-193).

Cholesterol has also been identified as a regulator of stearoyl-CoA desaturase gene expression by a mechanism involving repression of the maturation of the sterol regulatory element binding protein (Bene *et al.*, *Biochem. Biophys. Res. Commun.*, **2001**, *284*, 1194-1198; Ntambi, *J. Lipid Res.*, **1999**, *40*, 1549-1558).

Thiazolidinediones have been employed as regulators of stearoyl-CoA desaturase activity in murine 3T3-L1 adipocytes (Kim *et al.*, *J. Lipid Res.*, **2000**, *41*, 1310-1316), and in diabetic rodents (Singh Ahuja *et al.*, *Mol. Pharmacol.*, **2001**, *59*, 765-773).

Compositions comprising a saponin in an amount effective to inhibit stearoyl-CoA desaturase enzyme activity were described. The saponin was derived from a source selected from the group consisting of *Quillaja saponaria*, *Panax trifolium*, *Panax quinquefolium* and *Glycyrrhiza glabra* (Chavali and Forse, International patent publication No. WO 99/63979 **1999**).

An inhibitor of stearoyl-CoA desaturase was prepared in a form suitable for oral, parenteral, rectal or dermal administration for use in modifying the lipid structure of cell membranes. The inhibitor was described as consisting of a saturated fatty acid having from 12 to 28 carbon atoms in the alkyl chain, e.g. stearic acid, or a pharmaceutically acceptable derivative thereof prepared in a form suitable for parenteral, rectal or dermal administration (Wood *et al.*, European Patent No. EP 238198 **1987**). A stearoyl-CoA desaturase antisense vector has been used to reduce expression levels of stearoyl-CoA desaturase in chicken LMH hepatoma cells (Diot *et al.*, *Arch. Biochem. Biophys.*, **2000**, *380*, 243-250).

To date, investigative strategies aimed at inhibiting stearoyl-CoA desaturase function include the previously cited uses of polyunsaturated fatty acids, saturated fatty acids, thiazolidinediones, cholesterol, and an antisense vector. However, these strategies are untested as therapeutic protocols. Consequently, there remains a long felt need for additional agents capable of effectively inhibiting stearoyl-CoA desaturase.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of stearoyl-CoA desaturase. Such compositions and methods are shown to modulate the expression of stearoyl-CoA desaturase, including inhibition of both isoforms of stearoyl-CoA desaturase.

In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with

nucleic acids encoding stearoyl-CoA desaturase. Such compounds, particularly antisense oligonucleotides, are targeted to a nucleic acid encoding stearoyl-CoA desaturase, and modulate the expression of stearoyl-CoA desaturase. Pharmaceutical and other compositions comprising the compounds of the invention are also provided.

Further provided are methods of modulating the expression of stearoyl-CoA desaturase in cells or tissues comprising contacting the cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of stearoyl-CoA desaturase, by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding stearoyl-CoA desaturase, ultimately modulating the amount of stearoyl-CoA desaturase produced. This is accomplished by providing antisense compounds that specifically hybridize with one or more nucleic acids encoding stearoyl-CoA desaturase.

Antisense technology is emerging as an effective means of reducing the expression of specific gene products and is uniquely useful in a number of therapeutic, diagnostic and research applications involving modulation of stearoyl-CoA desaturase expression.

As used herein, the terms "target nucleic acid" and "nucleic acid encoding stearoyl-CoA desaturase" encompass DNA encoding stearoyl-CoA desaturase, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the

normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds that specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of stearoyl-CoA desaturase. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In one embodiment of the present invention, inhibition is a preferred form of modulation of gene expression and mRNA is a preferred target.

For example, in one embodiment of the present invention, the compounds of the present invention inhibit expression of stearoyl-CoA desaturaseby at least 10% as measured in a suitable assay, such as those described in the examples below. In another embodiment, the compounds of the present invention inhibit expression of stearoyl-CoA desaturase by at least 25%. In still another embodiment of the invention, the compounds of the present invention inhibit expression of stearoyl-CoA desaturase by at least 40%. In yet a further embodiment of this invention, the compounds of the present invention inhibit expression of stearoyl-CoA desaturase by at least 50%. In a further embodiment of this invention, the compounds of the present invention inhibit expression of stearoyl-CoA desaturase by at least 60%. In another embodiment of this invention, the compounds of the present invention inhibit expression of stearoyl-CoA desaturase by at least 70%. In still another embodiment of this invention, the compounds of this invention inhibit expression of stearoyl-CoA desaturase by at least 80%. In another

embodiment of this invention, the compounds of this invention inhibit expression of stearoyl-CoA desaturase by at least 90% or higher. Exemplary compounds are illustrated in Examples 15, and 17 to 24 below.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process as described herein begins with the identification of a nucleic acid sequence encoding stearoyl-CoA desaturase. This may be, for example, a cellular gene (or mRNA transcribed from the gene). The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, results. In one embodiment of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes has a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG; and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an

mRNA molecule transcribed from a gene encoding stearoyl-CoA desaturase, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is another embodiment of a region of the nucleic acid sequence encoding stearoyl-CoA desaturase which may be targeted effectively. Other target regions of this invention include the 5' untranslated region (5'UTR) of the nucleic acid sequence encoding stearoyl-CoA desaturase, known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene. Still another target region is the 3' untranslated region (3'UTR) of the nucleic acid sequence encoding stearoyl-CoA desaturase, known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage.

The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region of the nucleic acid sequence encoding stearoyl-CoA desaturase may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, of the nucleic acid sequence encoding stearoyl-CoA desaturase may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions of the nucleic acid sequence encoding stearoyl-CoA desaturase, due to rearrangements or deletions, are also preferred targets. In another embodiment of this invention, introns of the nucleic acid sequence encoding stearoyl-CoA desaturase can also be effective target regions for antisense compounds targeted, for example, to DNA or pre-mRNA of the nucleic acid sequence encoding stearoyl-CoA desaturase.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect. See, e.g., Tables 1-5 below.

For example, Tables 1 and 2 illustrate antisense oligonucleotides that hybridize to target regions of nucleotide 9 to 5100 of the nucleotide sequence of human stearoyl CoA desaturase SEQ ID NO: 3. In one embodiment, for example, desirable oligonucleotides target regions within nucleotides 13 to 71. In another example, desirable oligonucleotides target regions within nucleotides 178 to 247. In another example, desirable oligonucleotides target

regions within nucleotides 482 to 843. In another example, desirable oligonucleotides target regions within nucleotides 892 to 1064. In another example, desirable oligonucleotides target regions within nucleotides 1303-1502. In another example, desirable oligonucleotides target regions within nucleotides 1597 - 2233. In another example, desirable oligonucleotides target regions within nucleotides 2245-2589. In another example, desirable oligonucleotides target regions within nucleotides 2676-3278. In another example, desirable oligonucleotides target regions within nucleotides 3342 - 3499. In another example, desirable oligonucleotides target regions within nucleotides 3655-3674. In another example, desirable oligonucleotides target regions within nucleotides 3707-3790. In another example, desirable oligonucleotides target regions within nucleotides 3825-3853. In another example, desirable oligonucleotides target regions within nucleotides 3911-4072. In another example, desirable oligonucleotides target regions within nucleotides 4132-4224. In another example, desirable oligonucleotides target regions within nucleotides 4261-4398. In another example, desirable oligonucleotides target regions within nucleotides 4420-4554. In another example, desirable oligonucleotides target regions within nucleotides 4645-4677. In another example, desirable oligonucleotides target regions within nucleotides 4834-4865. In another example, desirable oligonucleotides target regions within nucleotides 4892-5100. Oligonucletides that target any nucleotide sequence within SEQ ID NO: 3, with the explicit exclusion of target regions between nucleotides 70-91, 242-262 and 860-882, are included within this invention.

As another example, Table 2 indicates illustrative oligonucleotides that hybridize to target regions found within nucleotides 505 to 14020 of the nucleotide sequence of human stearoyl CoA desaturase SEQ ID NO: 81.

Tables 3 - 5 illustrate oligonucleotides that bind to target regions within nucleotides 1 to 5366 of the nucleotide sequence of mouse stearoyl CoA desaturase SEQ ID NO: 222.

In the context of this invention, "hybridization" means

hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the target nucleic acid sequence (DNA or RNA) encoding stearoyl-CoA desaturase.

It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility of the stearoyl-CoA desaturase enzyme. There also must be a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-stearoyl-CoA desaturase target sequences under conditions in which specific binding is desired. Such conditions include physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, include conditions in which the assays are performed.

For example, in one embodiment, the antisense compounds of the present invention comprise at least 80% sequence

complementarity to a target region within the target nucleic acid of stearoyl-CoA desaturase to which they are targeted. In another embodiment, the antisense compounds of the present invention comprise at least 90% sequence complementarity to a target region within the target nucleic acid of stearoyl-CoA desaturase to which they are targeted. In still another embodiment of this invention, the antisense compounds of the present invention comprise at least 95% sequence complementarity to a target region within the target nucleic acid sequence of stearoyl-CoA desaturase to which they are targeted. For example, an antisense compound in which 18 of 20 nucleobases of the antisense compound are complementary, and would therefore specifically hybridize, to a target region would represent 90 percent complementarity. Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using basic local alignment search tools (BLAST programs) (Altschul *et al.*, *J. Mol. Biol.*, **1990**, 215, 403-410; Zhang and Madden, *Genome Res.*, **1997**, 7, 649-656).

Antisense and other compounds of the invention that hybridize to the target and inhibit expression of stearoyl-CoA desaturase are identified as taught herein. In one embodiment of this invention, the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention (see, e.g., Tables 1-5). The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting (see, e.g., Tables 1-5). Therefore another embodiment of the invention encompasses compounds that hybridize to these active sites.

In one embodiment of this invention, the term ``illustrative target region'' is defined as a nucleobase sequence of a target region of stearoyl-CoA desaturase, to which an active antisense compound is targeted. For example, an illustrative target region may be at least 8 or at least 15 nucleobases in length. In still another embodiment an illustrative target region is at least 25 nucleobases of the

nucleic acid sequence or molecule encoding stearoyl-CoA desaturase, to which an active antisense compound is targeted. In still another embodiment an illustrative target region is at 35 nucleobases. In yet another embodiment an illustrative target region is at least 50 nucleobases of the nucleic acid sequence or molecule encoding stearoyl-CoA desaturase, to which an active antisense compound is targeted. In still another embodiment an illustrative target region is at least 70 nucleobases. In another embodiment an illustrative target region is at least 80 nucleobases or more. In still another embodiments, the illustrative target regions consist of consecutive nucleobases of the lengths identified above.

Exemplary additional target regions include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative target regions (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly additional target regions are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative target regions (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases).

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds that affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000**, 480, 17-24; Celis, *et al.*, *FEBS Lett.*, **2000**, 480, 2-16), SAGE (serial analysis of gene expression)(Madden, *et al.*, *Drug Discov. Today*, **2000**, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999**, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97, 1976-81), protein arrays and proteomics (Celis, *et al.*, *FEBS Lett.*, **2000**, 480, 2-16; Jungblut, *et al.*, *Electrophoresis*, **1999**, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, *et al.*, *FEBS Lett.*, **2000**, 480, 2-16; Larsson, *et al.*, *J. Biotechnol.*, **2000**, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, *et al.*, *Anal. Biochem.*, **2000**, 286, 91-98; Larson, *et al.*, *Cytometry*, **2000**, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000**, 3, 316-21), comparative genomic hybridization (Carulli, *et al.*, *J. Cell Biochem. Suppl.*, **1998**, 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, *Eur. J.*

Cancer, **1999**, 35, 1895-904) and mass spectrometry methods (reviewed in (To, *Comb. Chem. High Throughput Screen*, **2000**, 3, 235-41).

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals, particularly mammals, and including man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides that hybridize to the target nucleic acid encoding stearoyl-CoA desaturase and modulate expression of that enzyme.

The antisense compounds in accordance with this invention preferably comprise from at least 8 nucleobases

(i.e. about 8 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides. In one embodiment, antisense compounds of this invention are antisense oligonucleotides of at least about 15 nucleobases in length. In another embodiment, antisense compounds of this invention comprise about 25 nucleobases in length. In still another embodiment, antisense compounds of this invention comprise about 35 nucleobases in length. In yet another embodiment, antisense compounds of this invention comprise about 40 nucleobases in length. In still another embodiment, antisense compounds of this invention comprise about 50 nucleobases in length. In another embodiment, antisense compounds of this invention comprise about 60 nucleobases in length. In still another embodiment, antisense compounds of this invention comprise about 70 nucleobases in length. In yet another embodiment, antisense compounds of this invention comprise about 80 nucleobases in length.

In other embodiments, exemplary antisense compounds include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense compounds (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly, in another embodiment, such antisense compounds include at least 12 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense compounds. In yet another embodiment, the antisense compound includes at least 25 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense compounds. In a further embodiment, the antisense compound includes at least 30 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense compounds. In yet another embodiment, the antisense compound includes at least 50 consecutive nucleobases from the 5'-

terminus of one of the illustrative antisense compounds. In still another embodiment, the antisense compound includes at least 60 or more consecutive nucleobases from the 5'-terminus of one of the illustrative antisense compounds.

Similarly in another embodiment antisense compounds are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense compounds (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). In another embodiment, such antisense compounds include at least 12 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense compounds. In yet another embodiment, the antisense compound includes at least 25 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense compounds. In a further embodiment, the antisense compound includes at least 30 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense compounds. In yet another embodiment, the antisense compound includes at least 50 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense compounds. In still another embodiment, the antisense compound includes at least 60 or more consecutive nucleobases from the 3'-terminus of one of the illustrative antisense compounds. One having skill in the art, once armed with the antisense compounds illustrated, and other teachings herein will be able, without undue experimentation, to identify further antisense compounds of this invention.

Specific sequences of particular exemplary target regions of stearoyl-CoA desaturase and representative antisense and other compounds of the invention, which hybridize to the target, and inhibit expression of the target, are identified below are set forth below in Tables 1-5. One of skill in the art will recognize that these serve to

illustrate and describe particular embodiments within the scope of the present invention. Once armed with the teachings of the illustrative target regions described herein may without undue experimentation identify further target regions, as described above. In addition, one having ordinary skill in the art using the teachings contained herein will also be able to identify additional compounds, including oligonucleotide probes and primers, that specifically hybridize to these illustrative target regions using techniques available to the ordinary practitioner in the art.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this

specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage, i.e. a single inverted nucleoside residue that may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patent Nos.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl

internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.Patent Nos.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and

5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*, *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. Patent No. 5,489,677, and the amide backbones of the above referenced U.S. Patent No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent No. 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, poly-alkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-

methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylamino-ethoxyethoxy (also known in the art as 2'-O-dimethylamino-ethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_2)_2$, also described in examples hereinbelow.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methelyne $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in International patent publication Nos. WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-$CH=CH_2$), 2'-O-allyl (2'-O-CH_2-$CH=CH_2$) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Patent Nos.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in The *Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, *Angewandte Chemie*,

International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent Nos. 3,687,808, as well as U.S. Patent Nos.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States Patent No. 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules,

polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Publication No. WO93/07883 (Application PCT/US92/09196, filed October 23, 1992) the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan *et al.*, *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan *et al.*, *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov *et al.*, *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk *et al.*, *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea *et al.*, *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan *et al.*, *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra *et al.*,

Biochim. Biophys. Acta, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, *J. Pharmacol. Exp.* Ther., **1996**, *277*, 923-937. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999), which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Patent Nos.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes

antisense compounds that are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Patent Nos.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by

reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S. Patent Nos.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable

salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in International Patent Publication Nos. WO 93/24510 to Gosselin *et al.*, published December 9, 1993 or WO 94/26764, and U.S. 5,770,713 to Imbach *et al.*

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylene-diamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*, 1-19). The base addition salts of the acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of

an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium,

ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder that can be treated by modulating the expression of stearoyl-CoA desaturase is treated by administering antisense compounds in accordance with this invention. Among such diseases or disorder are included, for example, cardiovascular disease, obesity, non-insulin-dependent diabetes mellitus, skin disease, hypertension, neurological diseases, immune disorders and cancer.

The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically to prevent such diseases or disorders, e.g., to prevent or delay infection, undue weight gain, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding stearoyl-CoA desaturase, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides

of the invention with a nucleic acid encoding stearoyl-CoA desaturase can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of stearoyl-CoA desaturase in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations that include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC,

distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999, which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers, surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate,. Preferred fatty acids include arachidonic acid, undecanoic acid, oleic

acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in U. S. Published Patent Application No. 2003/0040497 (Feb. 27, 2003) and its parent applications; U. S. Published Patent Application No. 2003/0027780 (Feb. 6, 2003) and its parent applications; and U. S. Patent Application No.

09/082,624 (filed May 21, 1998), each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions, which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as

foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug, which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers,

dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, cited above).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, cited above). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/ lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group:

nonionic, anionic, cationic and amphoteric (Rieger, cited above).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, cited above).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these

formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture has been reviewed in the literature (Idson, cited above). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint (Rosoff, cited above; Idson, cited above). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile, which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, cited above). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, *in: Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-

215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, cited above; Block, cited above). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene

glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides *et al.*, *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides *et al.*, **1994** cited above; Ho *et al.*, *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermo-labile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastro-intestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan

monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome that is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of

water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, cited above). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes that interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the

cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res. Commun.*, *1987*, *147*, 980-985).

Liposomes that are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, **1992**, *19*, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (*e.g.* as a solution or as an emulsion) were ineffective (Weiner *et al.*, *Journal of Drug Targeting*, **1992**, *2*, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis *et al.*, *Antiviral Research*, **1992**, *18*, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu *et al. S.T.P.Pharma. Sci.*, **1994**, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes. This latter term, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters, 1987, 223*, 42; Wu *et al.*, *Cancer Research*, **1993**, *53*, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, **1987**, *507*, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, **1988**, *85*, 6949). U.S.

Patent No. 4,837,028 and International Patent Publication No. WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-*sn*-dimyristoylphosphatidylcholine are disclosed in International Patent Publication No. WO 97/13499 (Lim *et al.*).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem. Soc. Jpn.*, **1980**, *53*, 2778) described liposomes comprising a nonionic detergent, $2C_{12}15G$, that contains a PEG moiety. Illum *et al.* (*FEBS Lett.*, **1984**, *167*, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (*FEBS Lett.*, **1990**, *268*, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (*Biochimica et Biophysica Acta*, **1990**, *1029*, 91) extended such observations to other PEG-derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and International Patent Publication No. WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in International Patent Publication

No. WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in International Patent Publication No. WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in International Patent Publication No. WO 96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. International Patent Publication No. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. International Patent Publication No. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets that are so highly deformable that they are easily able to penetrate through pores that are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.* they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, cited above).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The

quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, cited above).

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in* Therapeutic *Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty

acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, **1991**, cited above); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, *J. Pharm. Pharmacol.*, **1988**, *40*, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-10} alkyl esters thereof (*e.g.*, methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, , **1991**, p.92, cited above; Muranishi, *Critical Reviews in* Therapeutic *Drug Carrier Systems*, **1990**, *7*, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., **1992**, *44*, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's The *Pharmacological Basis of* Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, **1996**, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium

taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, **1991**, page 92, cited above; Swinyard, Chapter 39 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**, pages 782-783; Muranishi, *Critical Reviews in* Therapeutic *Drug Carrier Systems*, **1990**, *7*, 1-33; Yamamoto *et al.*, *J. Pharm. Exp.* Ther., **1992**, *263*, 25; Yamashita *et al.*, *J. Pharm. Sci.*, **1990**, *79*, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, *618*, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediamine tetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, **1991**, page 92, cited above; Muranishi, **1990**, cited above; Buur *et al.*, *J. Control Rel.*, **1990**, *14*, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, **1990**, cited above). This

class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, **1991**, page 92, cited above); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, **1987**, *39*, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, International Patent Publication No. WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is

coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, **1995**, *5*, 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6*, 177-183).

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration that do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration that do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, The *Merck Manual of Diagnosis and* Therapy, 15th Ed. **1987**, pp. 1206-1228, Berkow *et al.*, eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir,

may also be combined in compositions of the invention. See, generally, The *Merck Manual of Diagnosis and* Therapy, 15th Ed., Berkow *et al.*, eds., **1987**, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance

doses, ranging from 0.01 .g to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

EXAMPLES

EXAMPLE 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, *36*, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures

whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble

salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and

stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M)

in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was

extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and

the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160°C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na_2SO_4, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and

stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated. Residue obtained was chromatographed (ethyl

acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee *et al.*, International Patent Publication No. WO 94/02501). Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-([2-phthalmidoxy]ethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH_2-O-CH_2-N$(CH_2)_2$, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O^2-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy) ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylamino-ethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH_2Cl_2 (2x200 mL). The combined CH_2Cl_2 layers are washed with saturated $NaHCO_3$ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate.

Evaporation of the solvent followed by silica gel chromatography using MeOH:CH_2Cl_2:Et_3N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH_2Cl_2 (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

EXAMPLE 2 - Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.

Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published International Patent Publication Nos. WO 94/17093 and WO 94/02499, herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

EXAMPLE 3 - Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patent Nos. 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patent Nos. 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as

described in U.S. Patent No. 5,223,618, herein incorporated by reference.

EXAMPLE 4 - PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, *4*, 5-23. They may also be prepared in accordance with U.S. Patent Nos. 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

EXAMPLE 5 - Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me]

Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s

repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide

linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

EXAMPLE 6 - Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full-length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang *et al.*, *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

EXAMPLE 7 - Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-

cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

EXAMPLE 8 - Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

EXAMPLE 9 - Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following seven cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

T-24 cells:

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 µg/mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 µg/mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

HepG2 cells:

The human hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells were routinely cultured in Eagle's MEM supplemented with 10% fetal calf serum, non-essential amino acids, and 1 mM sodium pyruvate (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

AML12 cells:

AML12 (alpha mouse liver 12) cell line was established from hepatocytes from a mouse (CD1 strain, line MT42) transgenic for human TGF alpha. Cells are cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone, and 90%; 10% fetal bovine serum. For subculturing, spent medium is removed; and fresh media of 0.25% trypsin, 0.03% EDTA solution is added. Fresh trypsin solution (1 to 2 ml) is added and the culture is left to sit at room temperature until the cells detach.

Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded

into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

b.END cells:

The mouse brain endothelial cell line b.END was obtained from Dr. Werner Risau at the Max Plank Instititute (Bad Nauheim, Germany). b.END cells were routinely cultured in DMEM, high glucose (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 3000 cells/well for use in RT-PCR analysis.

Primary mouse hepatocytes:

Primary mouse hepatocytes were prepared from CD-1 mice purchased from Charles River Labs (Wilmington, MA) and were routinely cultured in Hepatoyte Attachment Media (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco/Life Technologies, Gaithersburg, MD), 250nM dexamethasone (Sigma), and 10nM bovine insulin (Sigma). Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 10000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells are plated onto 100 mm or other standard tissue culture plates coated with rat tail collagen (200µg/mL) (Becton Dickinson) and treated similarly using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated

with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 µL OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM™-1 medium containing 3.75 µg/mL LIPOFECTIN™ reagent (Gibco BRL) and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCA**TTCTGCCCCC**AAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments.

EXAMPLE 10 - Analysis of oligonucleotide inhibition of stearoyl-CoA desaturase expression

Antisense modulation of stearoyl-CoA desaturase expression can be assayed in a variety of ways known in the

art. For example, stearoyl-CoA desaturase mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, F.*M. et al.*, *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., **1996**. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of stearoyl-CoA desaturase can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to stearoyl-CoA desaturase can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., **1997**. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., **1997**.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., **1998**. Western blot (immunoblot) analysis is standard in the art and can be found

at, for example, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., **1997**. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., **1991**.

EXAMPLE 11 - Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura *et al.*, *Clin. Chem.*, **1996**, *42*, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS, 60 µL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 µL of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 µL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 µL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

EXAMPLE 12 - Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and

buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 100µL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 µL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

EXAMPLE 13 - Real-time Quantitative PCR Analysis of stearoyl-CoA desaturase mRNA Levels

Quantitation of stearoyl-CoA desaturase mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or VIC, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after

antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM $MgCl_2$, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLD™ reagent, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL total RNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™ reagent, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA

using RiboGreen™ reagent (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent from Molecular Probes. Methods of RNA quantification by RiboGreen™ reagent are taught in Jones, L.J., et al, *Analytical Biochemistry*, **1998**, *265*, 368-374.

In this assay, 175 µL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human stearoyl-CoA desaturase were designed to hybridize to a human stearoyl-CoA desaturase sequence, using published sequence information (GenBank accession number AF097514, incorporated herein as SEQ ID NO: 3). For human stearoyl-CoA desaturase the PCR primers were:
forward primer: GATCCCGGCATCCGAGA (SEQ ID NO: 4)
reverse primer: GGTATAGGAGCTAGAGATATCGTCCTG (SEQ ID NO: 5)
and the PCR probe was: FAM-CCAAGATGCCGGCCCACTTGC-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human GAPDH the PCR primers were:
forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 7)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 8) and the PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

EXAMPLE 14 - Northern blot analysis of stearoyl-CoA desaturase mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ reagent (TEL-TEST "B" Inc., Friendswood, TX). Total

RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 apparatus (Stratagene, Inc, La Jolla, CA) and then robed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human stearoyl-CoA desaturase, a human stearoyl-CoA desaturase specific probe was prepared by PCR using the forward primer GATCCCGGCATCCGAGA (SEQ ID NO: 4) and the reverse primer GGTATAGGAGCTAGAGATATCGTCCTG (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ apparatus and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

EXAMPLE 15 - Antisense inhibition of human stearoyl-CoA desaturase expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides was designed to target different regions of the human stearoyl-CoA desaturase RNA, using published sequence (GenBank accession number AF097514, incorporated herein as SEQ ID NO: 3). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most)

nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human stearoyl-CoA desaturase mRNA levels in HepG2 cells by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

Inhibition of human stearoyl-CoA desaturase mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
147899	5'UTR	3	9	GTCCGGTATTTCCTCAGCCC	N.D.	10
147900	5'UTR	3	72	CCGCGGTGCGTGGAGGTCCC	N.D.	11
147901	5'UTR	3	121	TACGCGCTGAGCCGCGGCGC	N.D.	12
147902	5'UTR	3	141	GCGGTTTCGAAGCCCGCCGG	N.D.	13
147903	Coding	3	311	CCTCCATTCTGCAGGACCCT	N.D.	14
147904	Coding	3	471	TCCCAAGTGTAGCAGAGACA	N.D.	15
147905	Coding	3	571	CTCCTGCTGTTATGCCCAGG	N.D.	16
147906	Coding	3	691	CACGGTGGTCACGAGCCCAT	N.D.	17
147907	Coding	3	771	CAGCCAACCCACGTGAGAGA	22	18
147908	Coding	3	824	GACAAGTCTAGCGTACTCCC	49	19
147909	Coding	3	1011	GTTCACCAGCCAGGTGGCAT	10	20
147910	Coding	3	1111	TGTGGAAGCCCTCACCCACA	0	21
147911	Coding	3	1171	AGTTGATGTGCCAGCGGTAC	6	22
147912	Stop Codon	3	1307	GGACCCCAAACTCAGCCACT	25	23
147913	3'UTR	3	1581	TGCCTGGGAGGCAATAAGGG	8	24
147914	3'UTR	3	1861	ATACATGCTAACTCTCTCCC	10	25
147915	3'UTR	3	1941	AAGTCCTCATTAGGTAGGCA	37	26
147916	3'UTR	3	2241	TGTAATGAGCAGCTCATGGA	0	27
147917	3'UTR	3	2616	TCAGTAACCTTCTCAAGCCC	0	28
147918	3'UTR	3	2980	GGAGCTGCCTGGACAGCAAG	16	29
147919	3'UTR	3	3011	TCAGTGACCCTGAGCATTCT	30	30
147920	3'UTR	3	3231	TGGCTGGCCCACTGGCTCAA	11	31
147921	3'UTR	3	3291	GCATGCCCTCTGGTTCTGAC	7	32

147922	3'UTR	3	3471	GCTTTGCAGTTCACCCTGAC	23	33
147923	3'UTR	3	3502	GTGGTATCTCAAATCCCAGG	0	34
147924	3'UTR	3	3791	TAGTCCAGGCTAACCCCTGT	0	35
147925	3'UTR	3	3851	GTGATCTTCCCTTAGATCCT	0	36
147926	3'UTR	3	4101	CTCAGCAGACACACTCCCAC	3	37
147927	3'UTR	3	4226	GCTAAGTTGTCAGCACACCC	0	38
147928	3'UTR	3	4406	AAGTTTCCAGAATGAAGCCC	25	39
147929	3'UTR	3	4571	AGAGAATACACCCAAGATAC	0	40
147930	3'UTR	3	4708	TAGTTAAGTGACTTGCCCAG	0	41
147931	3'UTR	3	4771	GCCCTTTGAGGTAGGTCAGT	4	42
147932	3'UTR	3	4921	CCATATAGACTAATGACAGC	10	43
147933	3'UTR	3	5021	CTGTATGTTTCCGTGGCAAT	11	44
168231	5'UTR	3	101	CTTGCACGCTAGCTGGTTGT	N.D.	45
168232	Coding	3	331	GCATCGTCTCCAACTTATCT	N.D.	46
168233	Coding	3	451	TAAGGATGATGTTTCTCCAG	N.D.	47
168234	Coding	3	526	CCCAAAGCCAGGTGTAGAAC	N.D.	48
168235	Coding	3	601	TGTAAGAGCGGTGGCTCCAC	N.D.	49
168236	Coding	3	661	CATTCTGGAATGCCATTGTG	N.D.	50
168237	Coding	3	731	TTATGAGGATCAGCATGTGT	N.D.	51
168238	Coding	3	861	CCTCTGGAACATCACCAGTT	N.D.	52
168239	Coding	3	901	GGATGAAGCACATCAGCAGC	N.D.	53
168240	Coding	3	936	TTCACCCCAGAAATACCAGG	N.D.	54
168241	Coding	3	1082	GAAACCAGGATATTCTCCCG	N.D.	55
168242	Coding	3	1151	TCACTGGCAGAGTAGTCATA	N.D.	56
168243	Coding	3	1261	TAATCCTGGCCAAGATGGCG	N.D.	57
168244	3'UTR	3	1401	TCATCATCTTTAGCATCCTG	N.D.	58
168245	3'UTR	3	1601	GCAAAGACTGACCAGCTGCT	N.D.	59
168246	3'UTR	3	1748	GACTACCCAGAAGATTCTGT	N.D.	60
168247	3'UTR	3	1881	CTTCCCTCATCCTTACATTC	N.D.	61
168248	3'UTR	3	1985	CCCGAGCCAGGAGAGAAAGG	N.D.	62
168249	3'UTR	3	2102	CTTCCCCAGCAGAGACCACT	N.D.	63
168250	3'UTR	3	2281	CCAATATCCTGAAGATGGCA	N.D.	64
168251	3'UTR	3	2481	CCCAACTAATTCCTCCTCTC	N.D.	65
168252	3'UTR	3	2541	TATAGATCCTGTCCCTCAGC	N.D.	66
168253	3'UTR	3	2631	CTCCCAATAACTCACTCAGT	N.D.	67
168254	3'UTR	3	2826	AAGAGATTCCTAACCCTGCC	N.D.	68
168255	3'UTR	3	2941	CACACAAAGGAGGCTGCCTG	N.D.	69
168256	3'UTR	3	3051	AAGTGGCAGCTAGCTCTACT	N.D.	70
168257	3'UTR	3	3321	CACCCTCACCAAGTAAGCAG	N.D.	71
168258	3'UTR	3	3401	TGCTTCTTCCCAGTGAGAAC	N.D.	72
168259	3'UTR	3	3941	ATCAAGCAGGCATCTGATGA	N.D.	73
168260	3'UTR	3	4052	CCCTCAGCCTGAGGTGCCAT	N.D.	74
168261	3'UTR	3	4357	ATAATCCTCCACTCAGGCCC	N.D.	75
168262	3'UTR	3	4431	CACTTAAGAAAAGCAGCCCT	N.D.	76
168263	3'UTR	3	4681	CAGCAAGTCAGTGGCACAGT	N.D.	77
168264	3'UTR	3	4971	GGCTAGTTATCCACCGCTTC	N.D.	78
168265	3'UTR	3	5044	CCCAATCACAGAAAAGGCAT	N.D.	79
168266	3'UTR	3	5081	AACTACTATATCCCACATAA	N.D.	80

As shown in Table 1, SEQ ID NOs 18, 19, 20, 23, 25, 26, 29, 30, 31, 33, 39, 43 and 44 demonstrated at least 10% inhibition of human stearoyl-CoA desaturase expression in this assay. The target sites to which these preferred sequences are complementary are herein referred to as "active

sites" and are therefore preferred sites for targeting by compounds of the present invention.

EXAMPLE 16 - Western blot analysis of stearoyl-CoA desaturase protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 µl/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to stearoyl-CoA desaturase is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ apparatus(Molecular Dynamics, Sunnyvale CA).

EXAMPLE 17 - Antisense inhibition of human stearoyl-CoA desaturase expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides was designed to target different regions of the human stearoyl-CoA desaturase RNA, using published sequence (GenBank accession number AF097514, incorporated herein as SEQ ID NO: 3 and nucleotides 7371062 to 7389569 of the nucleotide sequence with the GenBank accession number NT_030059.7, incorporated herein as SEQ ID NO: 81). The oligonucleotides are shown in Table 2. ``Target site'' indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate

(P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human stearoyl-CoA desaturase mRNA levels in HepG2 cells by quantitative real-time PCR as described in other examples herein. The positive control oligonucleotide is ISIS 18078 (**GTGCG**CGCGAGCCCG**AAATC**, SEQ ID NO: 82), a 2'-O-methoxyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone, which is targeted to human Jun-N-terminal kinase-2 (JNK2). Data are averages from two experiments and are shown in Table 2. If present, "N.D." indicates "no data".

Table 2

Inhibition of human stearoyl-CoA desaturase mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	Region	Target Seq ID	Target Site	SEQUENCE	% Inhib	Seq ID No	Control SEQ ID NO
300870	5'UTR	3	13	ccgtgtccggtatttcctca	53	83	82
300871	5'UTR	3	25	ggcaacgggtgaccgtgtcc	75	84	82
300872	5'UTR	3	42	atttaaaggctagagctggc	60	85	82
300873	5'UTR	3	52	cgagccgggaatttaaaggc	40	86	82
300874	5'UTR	3	178	gaggctccggagcggagttc	63	87	82
300875	5'UTR	3	215	ttggctctcggatgccggga	69	88	82
300876	Start Codon	3	228	gtgggccggcatcttggctc	54	89	82
300877	Coding	3	239	tcctgcagcaagtgggccgg	88	90	82
300878	Coding	3	253	agctagagatatcgtcctgc	82	91	82
300879	Coding	3	482	ccatacagggctcccaagtg	42	92	82
300880	Coding	3	513	gtagaacttgcaggtaggaa	57	93	82
300881	Coding	3	566	gctgttatgcccagggcact	93	94	82
300882	Coding	3	667	agacatcattctggaatgcc	76	95	82
300883	Coding	3	709	ctgaaaacttgtggtgggca	42	96	82
300884	Coding	3	715	gtgtttctgaaaacttgtgg	60	97	82
300885	Coding	3	821	aagtctagcgtactcccctt	69	98	82
300886	Coding	3	873	tttgtagtacctcctctgga	36	99	82
300887	Coding	3	1045	cataaggacgatatccgaag	52	100	82
300888	Stop Codon	3	1303	cccaaactcagccactcttg	50	101	82
300889	3'UTR	3	1347	aaacctctgcctggctggtt	87	102	82
300890	3'UTR	3	1381	gtagcattattcagtagtta	58	103	82
300891	3'UTR	3	1419	tactggaatgggttaacatc	42	104	82
300892	3'UTR	3	1484	tcagcttagcatcataaagg	71	105	82
300893	3'UTR	3	1597	agactgaccagctgcttgcc	45	106	82
300894	3'UTR	3	1613	gctggacactgagcaaagac	65	107	82

300895	3'UTR	3	1620	tttggaagctggacactgag	51	108	82
300896	3'UTR	3	1668	tctggagcaaagaccattcg	91	109	82
300897	3'UTR	3	1704	cttcaaagctcacaacagct	69	110	82
300898	3'UTR	3	1711	ccacctacttcaaagctcac	68	111	82
300899	3'UTR	3	1716	tcaagccacctacttcaaag	45	112	82
300900	3'UTR	3	1723	ctctagctcaagccacctac	57	113	82
300901	3'UTR	3	1814	tgtgtcaaatgaagttgctt	66	114	82
300902	3'UTR	3	1842	cccgacaatttacctgcttt	75	115	82
300903	3'UTR	3	1869	ttacattcatacatgctaac	22	116	82
300904	3'UTR	3	1915	tgtctgatcatggcgagagg	90	117	82
300905	3'UTR	3	1969	aaggaagcatgctatgtggt	87	118	82
300906	3'UTR	3	1976	ggagagaaaggaagcatgct	81	119	82
300907	3'UTR	3	2040	aactatatgttgcggcattg	59	120	82
300908	3'UTR	3	2781	tagatgttaacagagacccc	15	121	82
300909	3'UTR	3	2839	aatcagggtagtgaagagat	27	122	82
300910	3'UTR	3	2859	gggtagagccaggaatcaag	32	123	82
300911	3'UTR	3	3020	agcagtggttcagtgaccct	83	124	82
300912	3'UTR	3	3035	tactttcaaaagagaagcag	27	125	82
300913	3'UTR	3	3056	cgtgaaagtggcagctagct	81	126	82
300914	3'UTR	3	3122	ccttgtcttgagccatcagt	77	127	82
300915	3'UTR	3	3132	ggtttgccagccttgtcttg	78	128	82
300916	3'UTR	3	3222	cactggctcaacatgagcgc	71	129	82
300917	3'UTR	3	3238	tgctctgtggctggcccact	93	130	82
300918	3'UTR	3	3252	aataaaccctcttttgctct	52	131	82
300919	3'UTR	3	3259	gactgaaaataaaccctctt	54	132	82
300920	3'UTR	3	3342	agagcactgactcaggcggg	81	133	82
300921	3'UTR	3	3357	ttgcactgccagctgagagc	88	134	82
300922	3'UTR	3	3371	tacttctacaagcattgcac	83	135	82
300923	3'UTR	3	3383	actgtttcctcctacttcta	63	136	82
300924	3'UTR	3	3409	cttgcccttgcttcttccca	70	137	82
300925	3'UTR	3	3432	tttcgaggtgaggcacttgg	60	138	82
300926	3'UTR	3	3480	tcagccaaagctttgcagtt	77	139	82
300927	3'UTR	3	3655	ttctgctttgatgactgagc	86	140	82
300928	3'UTR	3	2052	atcctcggcctcaactatat	58	141	82
300929	3'UTR	3	2136	tccttgttattaaagaaaaa	35	142	82
300930	3'UTR	3	2146	ctaagaaatctccttgttat	28	143	82
300931	3'UTR	3	2162	cttcttgatatatgaactaa	11	144	82
300932	3'UTR	3	2171	acttcaagacttcttgatat	45	145	82
300933	3'UTR	3	2214	aaattccatgagctgctgtt	27	146	82
300934	3'UTR	3	2245	gaactgtaatgagcagctca	36	147	82
300935	3'UTR	3	2272	tgaagatggcagagcagaaa	27	148	82
300936	3'UTR	3	2321	tggaaatgccacagccatct	70	149	82
300937	3'UTR	3	2361	cgacttcacctccttaaatc	56	150	82
300938	3'UTR	3	2397	gcaatgtatatatgtatata	31	151	82
300939	3'UTR	3	2506	ccagcagtggagaggaaatt	27	152	82
300940	3'UTR	3	2525	cagcctctccatctcatgtc	61	153	82
300941	3'UTR	3	2570	ctatgtgaagttcgctctta	66	154	82
300942	3'UTR	3	2589	cgtgttctcagatcccttcc	0	155	82
300943	3'UTR	3	2676	aactaattaatgaatggacc	36	156	82
300944	3'UTR	3	2700	ttactcatttcaaggagaaa	54	157	82
300945	3'UTR	3	2715	gaagccttctagtttttact	55	158	82
300946	3'UTR	3	2726	cactgtggagagaagccttc	71	159	82

300947	3'UTR	3	2732	gcacaacactgtggagagaa	58	160	82
300948	3'UTR	3	3679	aatcttaatagagcaaagcc	0	161	82
300949	3'UTR	3	3707	gactgagtgtttggtagtgt	26	162	82
300950	3'UTR	3	3771	agcctctacgcaattaacac	38	163	82
300951	3'UTR	3	3825	ctgaggtgaatagctcaaaa	51	164	82
300952	3'UTR	3	3834	ccttttctactgaggtgaat	65	165	82
300953	3'UTR	3	3911	tagaaataccagcagacatt	37	166	82
300954	3'UTR	3	3993	gcacacgattacaataggaa	62	167	82
300955	3'UTR	3	3999	tccatggcacacgattacaa	64	168	82
300956	3'UTR	3	4004	tcagatccatggcacacgat	54	169	82
300957	3'UTR	3	4041	aggtgccatccagccttatg	12	170	82
300958	3'UTR	3	4053	gccctcagcctgaggtgcca	21	171	82
300959	3'UTR	3	4132	agctttagaatcttgaaaat	25	172	82
300960	3'UTR	3	4150	aatgtgtcacttgaattgag	26	173	82
300961	3'UTR	3	4193	ctgttagaaatccggactct	33	174	82
300962	3'UTR	3	4205	ccaaagcagggactgttaga	41	175	82
300963	3'UTR	3	4261	caacactgtgattagaaaag	20	176	82
300964	3'UTR	3	4321	cttcagtagggtctcaggtg	43	177	82
300965	3'UTR	3	4331	ctaccagccacttcagtagg	37	178	82
300966	3'UTR	3	4347	actcaggccccttttctac	34	179	82
300967	3'UTR	3	4364	gatactgataatcctccact	18	180	82
300968	3'UTR	3	4379	aatcctgcaaatcgtgatac	34	181	82
300969	3'UTR	3	4420	agcagccctaacaaaagttt	34	182	82
300970	3'UTR	3	4535	aaattttccattttaaatgc	23	183	82
300971	3'UTR	3	4578	cacttacagagaatacaccc	38	184	82
300972	3'UTR	3	4584	gagctacacttacagagaat	26	185	82
300973	3'UTR	3	4628	aacatggccacctcgctttt	16	186	82
300974	3'UTR	3	4645	gccttaaccaccagcataac	40	187	82
300975	3'UTR	3	4653	aggccctggccttaaccacc	60	188	82
300976	3'UTR	3	4658	tggagaggccctggccttaa	55	189	82
300977	3'UTR	3	4786	tcatgcctcaaaactgccct	34	190	82
300978	3'UTR	3	4800	ctaaaaagcattagtcatgc	0	191	82
300979	3'UTR	3	4834	agaattcctgtgctgaagga	13	192	82
300980	3'UTR	3	4841	ggtcttgagaattcctgtgc	47	193	82
300981	3'UTR	3	4846	actcaggtcttgagaattcc	43	194	82
300982	3'UTR	3	4868	ggacattcctattataaaaa	9	195	82
300983	3'UTR	3	4892	acacggacgtatcaagttca	41	196	82
300984	3'UTR	3	5024	cctctgtatgtttccgtggc	67	197	82
300985	exon	81	505	tgcgaggagttgactggcgc	39	198	82
300986	exon	81	512	ggcaaagtgcgaggagttga	45	199	82
300987	exon:intron	81	799	ggaaactcacatcgtcctgc	33	200	82
300988	exon:intron	81	1614	ggctgcttaccccaaagcca	29	201	82
300989	intron	81	2854	ctcagttgcatttcactgta	45	202	82
300990	intron	81	3557	gtgggaagagaagatgtcca	4	203	82
300991	intron	81	5287	gccttctctaaggttttaag	50	204	82
300992	intron:exon	81	5633	tagaataccctgccaggag	34	205	82
300993	exon:intron	81	5764	aacttcttacctggaatgcc	18	206	82
300994	intron	81	7232	ccttgcaaaagagctcatac	56	207	82
300995	exon:intron	81	7900	cttcactcacctcctctgga	0	208	82
300996	intron	81	8630	tttgcactgtctctccccac	13	209	82
300997	intron	81	8878	tcagtggtttcttacacttg	77	210	82
300998	intron:exon	81	9799	tttgtagtacctacattgac	4	211	82

300999	exon:intron	81	10032	gctgacttacccacagctcc	10	212	82
301000	intron	81	10163	tactgccccctaattttata	0	213	82
301001	intron	81	12377	ccatttgcgatacaggaaac	27	214	82
301002	intron:exon	81	14001	aagccctcacctgaaacaaa	22	215	82

As shown in Table 2, SEQ ID NOs 83, 84, 85, 87, 88, 89, 90, 91, 93, 94, 95, 97, 98, 100, 101, 102, 103, 105, 107, 108, 109, 110, 111, 113, 114, 115, 117, 118, 119, 120, 124, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 149, 150, 153, 154, 157, 158, 159, 160, 164, 165, 167, 168, 169, 188, 189, 197, 204, 207 and 210 demonstrated at least 50% inhibition of human stearoyl-CoA desaturase expression in this assay. Preferred antisense oligonucleotide sequences are SEQ ID NOs 94, 130, 140 and 134. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

EXAMPLE 18 - Antisense inhibition of mouse stearoyl-CoA desaturase expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides was designed to target different regions of the mouse stearoyl-CoA desaturase RNA, using published sequence (GenBank Accession number M21280.1, incorporated herein as SEQ ID NO: 216; GenBank Accession number M21281.1, incorporated herein as SEQ ID NO: 217; GenBank Accession number M21282.1, incorporated herein as SEQ ID NO: 218; GenBank Accession number M21283.1, incorporated herein as SEQ ID NO: 219; GenBank Accession number M21284.1, incorporated herein as SEQ ID NO: 220; GenBank Accession number M21285.1, incorporated herein as SEQ ID NO: 221; the concatenation of SEQ ID NOs 216, 217, 218, 219, 220 and 221, incorporated herein as SEQ ID NO: 222). The oligonucleotides are shown in Table 3. ``Target site`` indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 3 are

chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

Table 3

Chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap targeted to mouse stearoyl-CoA

ISIS #	Region	Target SEQ ID NO	Target Site	Sequence	SEQ ID NO
180548	5'UTR	222	9	agatctcttggagcatgtgg	223
180549	5'UTR	222	71	cttctctcgttcatttccgg	224
180550	5'UTR	222	126	cttctttcatttcaggacgg	225
180551	5'UTR	222	161	tccctcctcatcctgatagg	226
180552	5'UTR	222	191	cctccagacgtactccagct	227
180553	5'UTR	222	211	aggaccatgagaatgatgtt	228
180554	5'UTR	222	231	acaggcctcccaagtgcagc	229
180555	5'UTR	222	250	ggaaccagtatgatcccgta	230
180557	5'UTR	222	291	agtagaaaatcccgaagagg	231
180558	5'UTR	222	321	cggctgtgatgcccagagcg	232
180559	5'UTR	222	341	gctccagaggcgatgagccc	233
180560	5'UTR	222	361	cgagccttgtaagttctgtg	234
180561	5'UTR	222	391	gcaatgattaggaagatccg	235
180562	5'UTR	222	421	tacacgtcattttggaacgc	236
180563	5'UTR	222	441	ggtgatctcgggcccagtcg	237
180564	5'UTR	222	471	cgtgtgtttctgagaacttg	238
180565	5'UTR	222	591	cggctttcaggtcagacatg	239
180566	5'UTR	222	611	ctggaacatcaccagcttct	240
180567	5'UTR	222	648	agcacatcagcaggaggccg	241
180568	5'UTR	222	651	tgaagcacatcagcaggagg	242
180569	5'UTR	222	691	gtctcgccccagcagtacca	243
180570	5'UTR	222	741	gcaccagagtgtatcgcaag	244
180571	5'UTR	222	761	caccagccaggtggcgttga	245
180572	5'UTR	222	781	tagagatgcgcggcactgtt	246
180573	5'UTR	222	812	ttgaatgttcttgtcgtagg	247
180574	Start Codon	222	855	cctcgcccacggcacccagg	248
180575	Coding	222	869	gtagttgtggaagccctcgc	249
180576	Coding	222	881	gaaggtgtggtggtagttgt	250
180577	Coding	222	911	gcggtactcactggcagagt	251
180578	Coding	222	929	ggtgaagttgatgtgccagc	252
180579	Coding	222	1011	tcctggctaagacagtagcc	253

180580	Coding	222	1031	cccgtctccagttctcttaa	254
180581	Coding	222	1039	ttgtgactcccgtctccagt	255
180582	Coding	222	1049	tcagctactcttgtgactcc	256

In a further embodiment of the present invention, a series of oligonucleotides was designed to target different regions of the mouse stearoyl-CoA desaturase RNA, using published sequences (GenBank Accession number M21280.1, incorporated herein as SEQ ID NO: 216; GenBank Accession number M21281.1, incorporated herein as SEQ ID NO: 217; GenBank Accession number M21282.1, incorporated herein as SEQ ID NO: 218; GenBank Accession number M21283.1, incorporated herein as SEQ ID NO: 219; GenBank Accession number M21284.1, incorporated herein as SEQ ID NO: 220; GenBank Accession number M21285.1, incorporated herein as SEQ ID NO: 221; the concatenation of SEQ ID NOs 216, 217, 218, 219, 220 and 221, incorporated herein as SEQ ID NO: 222). The oligonucleotides are shown in Table 4. ``Target site'' indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 4 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

Probes and primers to mouse stearoyl-CoA desaturase were designed to hybridize to a mouse stearoyl-CoA desaturase sequence, using published sequence information (SEQ ID NO: 222). For mouse stearoyl-CoA desaturase the PCR primers were:

forward primer: ACACCAGAGACATGGGCAAGT (SEQ ID NO: 257)

reverse primer: CATCACACACTGGCTTCAGGAA (SEQ ID NO: 258) and the PCR probe was: FAM-CTGAAGTGAGGTCCATTAG-TAMRA (SEQ ID NO: 259) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-

Applied Biosystems, Foster City, CA) is the quencher dye. For mouse GAPDH the PCR primers were: forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO:260) reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO:261) and the PCR probe was: 5' JOE- CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID NO: 262) where JOE is the fluorescent reporter dye and TAMRA is the quencher dye.

The compounds were analyzed for their effect on mouse stearoyl-CoA desaturase mRNA levels in b.END cells by quantitative real-time PCR as described in other examples herein. The positive control oligonucleotide is ISIS 18078 (**GTGCG**CGCGAGCCCG**AAATC**, SEQ ID NO: 82), a 2'-O-methoxyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone, which is targeted to human Jun-N-terminal kinase-2 (JNK2). Data are averages from two experiments and are shown in Table 4. If present, "N.D." indicates "no data".

Table 4

Inhibition of mouse stearoyl-CoA desaturase mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

Isis #	Region	Target SEQ ID NO	Target Site	Sequence	% Inhib	SEQ ID NO	Control SEQ ID NO
185154	exon:intron	216	876	ggaagctcacctcttggagc	48	263	82
185155	exon:intron	217	269	ctgctcaccgaagagggcag	0	264	82
185156	intron:exon	218	1	gtagtagaaaatccctgcaa	6	265	82
185157	exon:intron	219	202	tcccttacctcctctggaac	0	266	82
185158	exon:intron	220	228	tgacttacccacggcaccca	41	267	82
185159	intron:exon	221	1	gtggaagccctcgcctgcaa	83	268	82
185160	5'UTR	222	68	ctggctaccgccactcacaa	0	269	82
185161	5'UTR	222	142	aagcctaggactttggtctg	51	270	82
185162	5'UTR	222	148	gtgtgcaagcctaggacttt	0	271	82
185163	5'UTR	222	156	taggaattgtgtgcaagcct	0	272	82
185164	5'UTR	222	275	atctgctgttccctctgcct	0	273	82
185165	5'UTR	222	445	tccagagtagaccttggagg	15	274	82
185166	5'UTR	222	571	ctagccaaggaagccaggcg	12	275	82
185167	5'UTR	222	581	gcagagatagctagccaagg	2	276	82
185168	5'UTR	222	612	ttttatcggctgccagcaaa	41	277	82
185169	5'UTR	222	644	ggatgaccgtgttcagtatt	41	278	82
185170	5'UTR	222	697	tggctgtgcacagatctcct	82	279	82
185171	5'UTR	222	708	tcagcccggtctggctgtgc	56	280	82

185172	5'UTR	222	748	gcgcttggaaacctgccctc	59	281	82
185173	5'UTR	222	768	tgtaggcgagtggcggaact	43	282	82
185174	5'UTR	222	795	gtggacttcggttccggagc	31	283	82
185175	5'UTR	222	830	ttgctcgcctcactttccca	79	284	82
185176	5'UTR	222	854	gtgggccggcatgatgatag	67	285	82
185177	5'UTR	222	877	gaactggagatctcttggag	51	286	82
185178	Coding	222	1150	tagaaaatcccgaagagggc	0	287	82
185179	Coding	222	1160	ggtcatgtagtagaaaatcc	10	288	82
185180	Coding	222	1165	gcgctggtcatgtagtagaa	40	289	82
185181	Coding	222	1676	ggattgaatgttcttgtcgt	81	290	82
185182	Coding	222	1681	tcccgggattgaatgttctt	46	291	82
185183	Coding	222	1688	gatattctcccgggattgaa	39	292	82
185184	Coding	222	1858	gtagccttagaaactttctt	52	293	82
185185	Stop Codon	222	1918	cccaaagctcagctactctt	65	294	82
185186	3'UTR	222	1934	aacaggaactcagaagccca	90	295	82
185187	3'UTR	222	1967	cagaatattaaatctctgcc	48	296	82
185188	3'UTR	222	1984	agttgttagttaatcaacag	0	297	82
185189	3'UTR	222	2159	aattgtatatgcatttatca	62	298	82
185190	3'UTR	222	2208	ctgtatagaatgttcaaatt	2	299	82
185191	3'UTR	222	2236	acagcatgttccttggcttt	51	300	82
185192	3'UTR	222	2246	tagcatcaaaacagcatgtt	46	301	82
185193	3'UTR	222	2259	accatgctcaccctagcatc	45	302	82
185194	3'UTR	222	2408	aaggatcagtatttcagaaa	39	303	82
185195	3'UTR	222	2552	tctctcgagacaatctactt	67	304	82
185196	3'UTR	222	2821	cttcagttaccaaaagctaa	37	305	82
185197	3'UTR	222	2887	aaatgtcagctgtttagtta	0	306	82
185198	3'UTR	222	3002	ggcaacccaggcaacacctc	39	307	82
185199	3'UTR	222	3017	gccacgaaagaaactggcaa	23	308	82
185200	3'UTR	222	3102	atgttccccaagggcttcat	86	309	82
185201	3'UTR	222	3112	tccctggcagatgttcccca	76	310	82
185202	3'UTR	222	3427	ctggctctgcttcctgaagc	48	311	82
185203	3'UTR	222	3569	gctgagctgttaactcacaa	71	312	82
185204	3'UTR	222	3640	cacacaccgagacagatcaa	79	313	82
185205	3'UTR	222	3828	caggaagcagaccctcttcc	42	314	82
185206	3'UTR	222	3958	aatactgatgtgatgttttc	65	315	82
185207	3'UTR	222	3968	atggttctaaaatactgatg	36	316	82
185208	3'UTR	222	4046	actgagtgtttggcacctta	79	317	82
185209	3'UTR	222	4066	ggctctgattctacaagtga	61	318	82
185210	3'UTR	222	4116	tcaacaaaacagctcagagc	83	319	82
185211	3'UTR	222	4127	gattttctacttcaacaaaa	56	320	82
185212	3'UTR	222	4333	cttaaagacaccaggacctc	56	321	82
185213	3'UTR	222	4387	catctggaaactgttataaa	45	322	82
185214	3'UTR	222	4466	ctaagggaaggagtgagact	42	323	82
185215	3'UTR	222	4608	ttacttcccaccaaatttga	59	324	82
185216	3'UTR	222	4652	tgacaatgataacgaggacg	81	325	82
185217	3'UTR	222	4760	cagatggtggctttgctaac	0	326	82
185218	3'UTR	222	4825	ttgttacaagagaaaggata	68	327	82
185219	3'UTR	222	4884	tcagatacttagcccaggag	74	328	82
185220	3'UTR	222	4902	tgttgagatgtgagactgtc	50	329	82
185221	3'UTR	222	5010	cacctcagaactgcccttga	73	330	82
185222	3'UTR	222	5018	gctctaatcacctcagaact	84	331	82
185223	3'UTR	222	5074	ggagtctgtatgaatacctc	64	332	82

185224	3'UTR	222	5132	tctctgggaagagcaatgta	58	333	82
185225	3'UTR	222	5170	gtaggtagtcttgcactttg	36	334	82
185226	3'UTR	222	5211	aggaagggaaaggtttcctg	38	335	82
185227	3'UTR	222	5268	tacacttgggtcacaaataa	49	336	82
185228	3'UTR	222	5280	aatcatccaaattacacttg	50	337	82
185229	3'UTR	222	5303	cttcaagagttgatattaat	60	338	82
185230	3'UTR	222	5329	atacaatctcaatcagtaca	76	339	82
185231	3'UTR	222	5347	cacttttattaggaacaaat	0	340	82

As shown in Table 4, SEQ ID NOs 263, 267, 268, 270, 277, 278, 279, 280, 281, 282, 284, 285, 286, 289, 290, 291, 293, 294, 295, 296, 298, 300, 301, 302, 304, 309, 310, 311, 312, 313, 314, 315, 317, 318, 319, 320, 321, 322, 323, 324, 325, 327, 328, 329, 330, 331, 332, 333, 336, 337, 338, 339 demonstrated at least 40% inhibition of stearoyl-CoA desaturase in this experiment and are therefore preferred. More preferred are SEQ ID NOs 295, 331 and 268. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention.

In a further embodiment of the present invention, a series of oligonucleotides was designed to target different regions of the mouse stearoyl-CoA desaturase RNA, using published sequences (GenBank Accession number M21280.1, incorporated herein as SEQ ID NO: 216; GenBank Accession number M21281.1, incorporated herein as SEQ ID NO: 217; GenBank Accession number M21282.1, incorporated herein as SEQ ID NO: 218; GenBank Accession number M21283.1, incorporated herein as SEQ ID NO: 219; GenBank Accession number M21284.1, incorporated herein as SEQ ID NO: 220; GenBank Accession number M21285.1, incorporated herein as SEQ ID NO: 221; the concatenation of SEQ ID NOs 216, 217, 218, 219, 220 and 221, incorporated herein as SEQ ID NO: 222). The oligonucleotides are shown in Table 5. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 5 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten

2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

Probes and primers to mouse stearoyl-CoA desaturase were designed to hybridize to a mouse stearoyl-CoA desaturase sequence, using published sequence information (SEQ ID NO: 222). For mouse stearoyl-CoA desaturase the PCR primers were:
forward primer: TTCCGCCACTCGCCTACA (SEQ ID NO: 341)
reverse primer: CTTTCCCAGTGCTGAGATCGA (SEQ ID NO: 342) and the PCR probe was: FAM- CAACGGGCTCCGGAACCGAA-TAMRA (SEQ ID NO: 343) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.
For mouse GAPDH the PCR primers were:
forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO:261)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO:262) and the PCR probe was: 5' JOE- CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID NO: 263) where JOE is the fluorescent reporter dye and TAMRA is the quencher dye.

The compounds were analyzed for their effect on mouse stearoyl-CoA desaturase mRNA levels in primary mouse hepatocytes by quantitative real-time PCR as described in other examples herein. The positive control oligonucleotide is ISIS 18078 (**GTGCG**CGCGAGCCCG**AAATC**, SEQ ID NO: 82), a 2'-O-methoxyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone, which is targeted to human Jun-N-terminal kinase-2 (JNK2). Data are averages from two experiments and are shown in Table 5. If present, "N.D." indicates "no data".

Table 5

Inhibition of mouse stearoyl-CoA desaturase mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

Isis #	Region	Target SEQ ID NO	Target Site	Sequence	% Inhib	SEQ ID NO	Control SEQ ID NO
180556	5'UTR	222	261	gcttgcaggagggaaccagt	40	344	82
244459	5'UTR	222	138	ctaggactttggtctggcgc	6	345	82
244461	5'UTR	222	280	gcgcaatctgctgttccctc	0	346	82
244464	5'UTR	222	401	agggcgcgctgctccaaccc	0	347	82
244467	5'UTR	222	462	aagaaagccaagtagattcc	0	348	82
244470	5'UTR	222	692	gtgcacagatctcctgggct	35	349	82
244472	5'UTR	222	736	ctgccctcctgactctcggg	54	350	82
244476	Coding	222	878	agaactggagatctcttgga	71	351	82
244479	Coding	222	1020	cctcctcatcctgataggtg	16	352	82
244481	Coding	222	1045	acgtactccagcttgggcgg	50	353	82
244484	Coding	222	1057	atgttcctccagacgtactc	33	354	82
244487	Coding	222	1062	gaatgatgttcctccagacg	43	355	82
244490	Coding	222	1068	ccatgagaatgatgttcctc	50	356	82
244493	Coding	222	1098	tcccgtacaggcctcccaag	54	357	82
244495	Coding	222	1128	tgtagagcttgcaggaggga	10	358	82
244498	Coding	222	1264	gccatggtgttggcaatgat	41	359	82
244501	Coding	222	1324	tctgagaacttgtggtgggc	18	360	82
244504	Coding	222	1329	gtgtttctgagaacttgtgg	50	361	82
244507	Coding	222	1334	ggcgtgtgtttctgagaact	63	362	82
244510	Coding	222	1347	aattgtgagggtcggcgtgt	11	363	82
244514	Coding	222	1357	ccacggcgggaattgtgagg	47	364	82
244517	Coding	222	1363	aagaagccacggcgggaatt	16	365	82
244520	Coding	222	1387	agcagccaacccacgtgaga	51	366	82
244523	Coding	222	1395	tgcgcacaagcagccaaccc	67	367	82
244526	Coding	222	1400	gtgtttgcgcacaagcagcc	52	368	82
244528	Coding	222	1408	acagccgggtgtttgcgcac	63	369	82
244532	Coding	222	1413	ctttgacagccgggtgtttg	3	370	82
244535	Coding	222	1418	cttctctttgacagccgggt	50	371	82
244538	Coding	222	1423	ccgcccttctctttgacagc	64	372	82
244541	Coding	222	1435	atgtccagttttccgcccttt	55	373	82
244542	Coding	222	1440	cagacatgtccagttttccg	55	374	82
244546	Coding	222	1445	caggtcagacatgtccagtt	48	375	82
244549	Coding	222	1450	gctttcaggtcagacatgtc	49	376	82
244553	Coding	222	1455	tctcggctttcaggtcagac	50	377	82
244554	Coding	222	1460	cagcttctcggctttcaggt	37	378	82
244557	Coding	222	1465	atcaccagcttctcggcttt	20	379	82
244560	Coding	222	1470	ggaacatcaccagcttctcg	42	380	82
244565	Coding	222	1477	ctcctctggaacatcaccag	17	381	82
244567	Coding	222	1486	ttgtagtacctcctctggaa	0	382	82
244569	Coding	222	1516	aggatgaagcacatcagcag	29	383	82
244572	Coding	222	1525	agcgtgggcaggatgaagca	45	384	82

244577	Coding	222	1538	gtaccagggcaccagcgtgg	37	385	82
244578	Coding	222	1543	cagcagtaccagggcaccag	25	386	82
244581	Coding	222	1548	cgccccagcagtaccagggc	13	387	82
244585	Coding	222	1583	gaaggtgctaacgaacaggc	18	388	82
244589	Coding	222	1627	ctgttcaccagccaggtggc	37	389	82
244591	Coding	222	1633	gcggcactgttcaccagcca	3	390	82
244595	Coding	222	1693	accaggatattctcccggga	62	391	82
244598	Coding	222	1732	tggtagttgtggaagccctc	54	392	82
244599	Coding	222	1768	tactcactggcagagtagtc	19	393	82
244602	Coding	222	1773	agcggtactcactggcagag	31	394	82
244607	Coding	222	1778	gtgccagcggtactcactgg	5	395	82
244609	Coding	222	1783	ttgatgtgccagcggtactc	29	396	82
244613	Coding	222	1792	gtggtgaagttgatgtgcca	40	397	82
244615	Coding	222	1798	aagaacgtggtgaagttgat	12	398	82
244619	Coding	222	1860	cagtagccttagaaactttc	75	399	82
244620	Coding	222	1885	ccagttctcttaatcctggc	63	400	82
244623	Coding	222	1891	ccgtctccagttctcttaat	37	401	82
244626	3'UTR	222	2006	taacaccccgatagcaatat	59	402	82
244630	3'UTR	222	2365	gagggtggacagacacaggc	4	403	82
244633	3'UTR	222	2445	cttgaagctaggaacaagga	68	404	82
244636	3'UTR	222	2647	tatggctacctctctctctc	82	405	82
244639	3'UTR	222	2920	ttttcatagtttcacaccat	47	406	82
244643	3'UTR	222	2970	tattttctaagtgaaatagt	5	407	82
244644	3'UTR	222	3243	taggcagcactaggcaggct	33	408	82
244647	3'UTR	222	3373	aggaacaggcctggacagca	36	409	82
244650	3'UTR	222	4168	gagggctataggtcagtaga	34	410	82
244655	3'UTR	222	4329	aagacaccaggacctcaatg	18	411	82
244656	3'UTR	222	4532	ccaatgtactgatgactctc	62	412	82
244660	3'UTR	222	4737	tcacaccacctcactggagc	62	413	82
244663	3'UTR	222	4987	agtaggtcagtattaataac	35	414	82
244667	3'UTR	222	5220	atctcattcaggaagggaaa	0	415	82
244668	3'UTR	222	5272	aaattacacttgggtcacaa	57	416	82
244673	3'UTR	222	5326	caatctcaatcagtacaagt	37	417	82

As shown in Table 5, SEQ ID NOs 344, 350, 351, 353, 355, 356, 357, 359, 361, 362, 364, 366, 367, 368, 369, 371, 372, 373, 374, 375, 376, 377, 380, 384, 391, 392, 397, 399, 400, 402, 404, 405, 406, 412, 413, 416 exhibited at least 40% inhibition of stearoyl-CoA desaturase in this experiment. A more preferred sequence is SEQ ID NO: 373. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" are therefore preferred for targeting by compounds of the present invention.

EXAMPLE 19 - Effects of antisense inhibition of mouse stearoyl-CoA desaturase expression in mice: mRNA levels in liver and fat tissue

Ob/ob mice harbor a mutation in the leptin gene. The leptin mutation on a C57Bl/6 background yields a db/db phenotype, characterized by, hyperglycemia, obesity, hyperlipidemia, and insulin resistance. However, a mutation in the leptin gene on a different mouse background can produce obesity without diabetes, and these mice are referred to as ob/ob mice. Leptin is a hormone that regulates appetite. Leptin deficiency results in obesity in animals and humans.

In accordance with the present invention, ISIS 185222 (SEQ ID NO: 332) was further investigated for its ability to reduce target levels in liver and fat tissue in ob/ob mice maintained on a high-fat (11% kcal from fat) or low-fat (2% from fat) diet.

ISIS 141923 (CCTTCCCTGAAGGTTCCTCC, SEQ ID NO: 418) is a scrambled control oligonucleotide. ISIS 141923 is a chimeric oligonucleotide ("gapmer") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

Eight-week old male ob/ob mice were dosed twice weekly by intraperitoneal injection with saline or 25 mg/kg of ISIS 185222 or ISIS 141923. Mice were maintained on a low-fat or high-fat diet. At the end of the ten-week investigation period, mice were sacrificed and evaluated for stearoyl-CoA desaturase and stearoyl-CoA desaturase-2 mRNA levels in liver and fat tissue. Inhibition of mRNA expression was determined by quantitative real-time PCR as described in other examples herein. The data are the averages of mRNA levels from nine mice per group and are presented in Table 6.

Table 6

Antisense inhibition of stearoyl-CoA desaturase in mouse liver and fat tissue

		Percent Inhibition					
		Saline control		ISIS 185222		ISIS 141923	
mRNA	Diet	Liver	Fat	Liver	Fat	Liver	Fat
stearoyl-CoA desaturase	High-fat	0	0	93	96	29	28
	Low-fat	0	0	94	98	0	0
stearoyl-CoA desaturase-2	High-fat	0	0	37	40	52	5
	Low-fat	0	0	37	0	0	0

The data demonstrate that the oligonucleotide of the present invention can inhibit the expression of stearoyl-CoA desaturase *in vivo*, in both liver and fat tissues. The data also suggest that antisense inhibition of stearoyl-CoA desaturase can reduce expression of stearoyl-CoA desaturase-2.

EXAMPLE 20 - Effects of antisense inhibition of stearoyl-CoA desaturase in a mouse model of obesity: organ weights and levels of serum cholesterol, triglyceride and liver enzymes

In accordance with the present invention, further investigation of the effects antisense inhibition of stearoyl-CoA desaturase was conducted in ob/ob mice. The saline-treated and antisense oligonucleotide-treated ob/ob mice described in Example 19 were also evaluated for body organ weight, levels of serum cholesterol and triglyceride and levels of liver enzymes ALT and AST at the end of the ten-week investigation period. Increased levels of ALT and AST are indicative of impaired liver function. Blood samples were collected and evaluated for cholesterol, triglyceride, ALT and AST levels. White adipose tissue (WAT), spleen and liver were individually weighed. Data are expressed as percent change relative to the saline control for the respective diet. The data represent the average of nine mice

per treatment group and are presented in Table 7.

Table 7

Effects of antisense inhibtion of stearoyl-CoA desaturase on cholesterol, triglyceride, ALT, AST and organ weight

		Percent Change						
		Liver Enzymes				Organ Weight		
		ALT	AST	CHOL	TRIG	Liver	Spleen	WAT
ISIS 185222	High-fat	-76	-72	-11	3	-8	19	2
	Low-fat	-60	-55	15	29	-29	-19	-4
ISIS 141923	High-fat	-42	-39	-1	-10	-4	20	9
	Low-fat	45	34	50	70	31	-41	27

The data demonstrate that, concomitant with reducing target mRNA expression (shown in Example 19), the oligonucleotide of the present invention lowers the levels of the liver enzyme ALT and AST in animals maintained on either a high-fat or low-fat diet, which is indicative of improved liver function. Histologically, mice treated with ISIS 185222 and maintained on a low-fat diet exhibit lowered hepatic fatty degeneration.

EXAMPLE 21 - Effects of antisense inhibition of stearoyl-CoA desaturase in a mouse model of obesity: plasma glucose and insulin, body weight, food consumption and oxygen consumption

In accordance with the present invention, the ob/ob mice described in Example 19 were further evaluated to assess the effects of antisense inhibition of stearoyl-CoA desaturase.

Mice were evaluated for plasma glucose and oxygen consumption following three weeks of treatment. The glucose evaluation was conducted following an overnight fast. The oxygen consumption was determined by measuring metabolic rate (MR) and respiratory quotient (RER) during both light and dark cycles. Plasma glucose and insulin (both in non-fasting mice), food consumption, oxygen consumption and total body weight were measured throughout the ten-week treatment

period. Shown in Table 8 are plasma insulin (non-fasting) following five weeks of treatment, food consumption following six weeks of treatment and plasma glucose (non-fasting) and total body weight following seven weeks of treatment. The data are the averages of measurements from seven to nine mice and are expressed as percent change relative to saline control for the respective diet. The data are presented in Table 8.

Table 8

Effects of antisense inhibtion of stearoyl-CoA desaturase on body weight, food consumption, insulin, glucose and oxygen consumption

		Percent Change								
		Weight		Insulin	Glucose		Oxygen consumption			
							MR		RER	
		Total Body	Food Consumed	Fed	Fed	Fast	Dark	Light	Dark	Light
ISIS 185222	High-fat	-3	-10	-3	2	-3	3	0	0	2
	Low-fat	-60	-55	15	29	-29	4	11	-6	0
ISIS 141923	High-fat	-42	-39	-1	-10	-4	-5	-5	3	0
	Low-fat	45	34	50	70	31	6	5	2	0

The data suggest that body weight and food consumption are lowered by treatment of ob/ob mice with the oligonucleotide of the present invention. Comparison of blood glucose, insulin and oxygen consumption in mice fed the same diet does not reveal any significant changes between saline-treated and antisense oligonucleotide-treated mice.

EXAMPLE 22 - RNA Synthesis

In general, RNA synthesis chemistry is based on the selective incorporation of various protecting groups at strategic intermediary reactions. Although one of ordinary skill in the art will understand the use of protecting groups in organic synthesis, a useful class of protecting groups

includes silyl ethers. In particular bulky silyl ethers are used to protect the 5′-hydroxyl in combination with an acid-labile orthoester protecting group on the 2′-hydroxyl. This set of protecting groups is then used with standard solid-phase synthesis technology. It is important to lastly remove the acid labile orthoester protecting group after all other synthetic steps. Moreover, the early use of the silyl protecting groups during synthesis ensures facile removal when desired, without undesired deprotection of 2′ hydroxyl.

Following this procedure for the sequential protection of the 5′-hydroxyl in combination with protection of the 2′-hydroxyl by protecting groups that are differentially removed and are differentially chemically labile, RNA oligonucleotides were synthesized.

RNA oligonucleotides are synthesized in a stepwise fashion. Each nucleotide is added sequentially (3′- to 5′-direction) to a solid support-bound oligonucleotide. The first nucleoside at the 3′-end of the chain is covalently attached to a solid support. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are added, coupling the second base onto the 5′-end of the first nucleoside. The support is washed and any unreacted 5′-hydroxyl groups are capped with acetic anhydride to yield 5′-acetyl moieties. The linkage is then oxidized to the more stable and ultimately desired P(V) linkage. At the end of the nucleotide addition cycle, the 5′-silyl group is cleaved with fluoride. The cycle is repeated for each subsequent nucleotide.

Following synthesis, the methyl protecting groups on the phosphates are cleaved in 30 minutes utilizing 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (S_2Na_2) in DMF. The deprotection solution is washed from the solid support-bound oligonucleotide using water. The support is then treated with 40% methylamine in water for 10 minutes at 55 °C. This releases the RNA oligonucleotides into solution, deprotects the exocyclic amines, and modifies the 2′- groups.

The oligonucleotides can be analyzed by anion exchange HPLC at this stage.

The 2´-orthoester groups are the last protecting groups to be removed. The ethylene glycol monoacetate orthoester protecting group developed by Dharmacon Research, Inc. (Lafayette, CO), is one example of a useful orthoester protecting group, which has the following important properties. It is stable to the conditions of nucleoside phosphoramidite synthesis and oligonucleotide synthesis. However, after oligonucleotide synthesis the oligonucleotide is treated with methylamine, which not only cleaves the oligonucleotide from the solid support but also removes the acetyl groups from the orthoesters. The resulting 2-ethyl-hydroxyl substituents on the orthoester are less electron withdrawing than the acetylated precursor. As a result, the modified orthoester becomes more labile to acid-catalyzed hydrolysis. Specifically, the rate of cleavage is approximately 10 times faster after the acetyl groups are removed. Therefore, this orthoester possesses sufficient stability in order to be compatible with oligonucleotide synthesis and yet, when subsequently modified, permits deprotection to be carried out under relatively mild aqueous conditions compatible with the final RNA oligonucleotide product.

Additionally, methods of RNA synthesis are well known in the art (Scaringe, S. A. Ph.D. Thesis, University of Colorado, 1996; Scaringe, S. A., *et al.*, *J. Am. Chem. Soc.*, **1998**, *120*, 11820-11821; Matteucci, M. D. and Caruthers, M. H. *J. Am. Chem. Soc.*, **1981**, *103*, 3185-3191; Beaucage, S. L. and Caruthers, M. H. *Tetrahedron Lett.*, **1981**, *22*, 1859-1862; Dahl, B. J., *et al.*, *Acta Chem. Scand,*. **1990**, *44*, 639-641; Reddy, M. P., *et al.*, *Tetrahedrom Lett.*, **1994**, *25*, 4311-4314; Wincott, F. *et al.*, *Nucleic Acids Res.*, **1995**, *23*, 2677-2684; Griffin, B. E., *et al.*, *Tetrahedron*, **1967**, *23*, 2301-2313; Griffin, B. E., *et al.*, *Tetrahedron*, **1967**, *23*, 2315-2331).

RNA antisense compounds (RNA oligonucleotides) of the present invention can be synthesized by the methods herein or

purchased from Dharmacon Research, Inc (Lafayette, CO). Once synthesized, complementary RNA antisense compounds can then be annealed by methods known in the art to form double stranded (duplexed) antisense compounds. For example, duplexes can be formed by combining 30 μl of each of the complementary strands of RNA oligonucleotides (50 uM RNA oligonucleotide solution) and 15 μl of 5X annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate) followed by heating for 1 minute at 90°C, then 1 hour at 37°C. The resulting duplexed antisense compounds can be used in kits, assays, screens, or other methods to investigate the role of a target nucleic acid.

EXAMPLE 23 - Design and screening of duplexed antisense compounds targeting stearoyl-CoA desaturase

In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements can be designed to target stearoyl-CoA desaturase. The nucleobase sequence of the antisense strand of the duplex comprises at least a portion of an oligonucleotide in Table 1. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini.

For example, a duplex comprising an antisense strand having the sequence CGAGAGGCGGACGGGACCG and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

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    cgagaggcggacgggaccgTT      Antisense Strand
    ||||||||||||||||||||
TTgctctccgcctgccctggc          Complement
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RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacon Research Inc.,

(Lafayette, CO). Once synthesized, the complementary strands are annealed. The single strands are aliquoted and diluted to a concentration of 50 uM. Once diluted, 30 µL of each strand is combined with 15µL of a 5X solution of annealing buffer. The final concentration of the buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2mM magnesium acetate. The final volume is 75 µL. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The tube is allowed to sit for 1 hour at 37°C at which time the dsRNA duplexes are used in experimentation. The final concentration of the dsRNA duplex is 20 µM. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate stearoyl-CoA desaturase expression.

When cells reached 80% confluency, they are treated with duplexed antisense compounds of the invention. For cells grown in 96-well plates, wells are washed once with 200 µL OPTI-MEM-1 reduced-serum medium (Gibco BRL) and then treated with 130 .L of OPTI-MEM-1 medium containing 12 µg/mL LIPOFECTIN reagent (Gibco BRL) and the desired duplex antisense compound at a final concentration of 200 nM. After 5 hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16 hours after treatment, at which time RNA is isolated and target reduction measured by RT-PCR.

EXAMPLE 24 - Design of phenotypic assays and *in vivo* studies for the use of stearoyl-CoA desaturase inhibitors

Phenotypic assays

Once stearoyl-CoA desaturase inhibitors have been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition. Phenotypic assays, kits and reagents for their use are well known to those skilled in the art and are herein used to

investigate the role and/or association of stearoyl-CoA desaturase in health and disease. Representative phenotypic assays, which can be purchased from any one of several commercial vendors, include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based assays including enzymatic assays (Panvera, LLC, Madison, WI; BD Biosciences, Franklin Lakes, NJ; Oncogene Research Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for obesity studies) are treated with stearoyl-CoA desaturase inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status, which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest.

Analysis of the geneotype of the cell (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the efficacy or potency of the stearoyl-CoA desaturase inhibitors. Hallmark genes, or those genes suspected to be associated with a

specific disease state, condition, or phenotype, are measured in both treated and untreated cells.

In vivo studies

The individual subjects of the *in vivo* studies described herein are warm-blooded vertebrate animals, which includes humans.

The clinical trial is subjected to rigorous controls to ensure that individuals are not unnecessarily put at risk and that they are fully informed about their role in the study. To account for the psychological effects of receiving treatments, volunteers are randomly given placebo or stearoyl-CoA desaturase inhibitor. Furthermore, to prevent the doctors from being biased in treatments, they are not informed as to whether the medication they are administering is a stearoyl-CoA desaturase inhibitor or a placebo. Using this randomization approach, each volunteer has the same chance of being given either the new treatment or the placebo.

Volunteers receive either the stearoyl-CoA desaturase inhibitor or placebo for eight week period with biological parameters associated with the indicated disease state or condition being measured at the beginning (baseline measurements before any treatment), end (after the final treatment), and at regular intervals during the study period. Such measurements include the levels of nucleic acid molecules encoding stearoyl-CoA desaturase or stearoyl-CoA desaturase protein levels in body fluids, tissues or organs compared to pre-treatment levels. Other measurements include, but are not limited to, indices of the disease state or condition being treated, body weight, blood pressure, serum titers of pharmacologic indicators of disease or toxicity as well as ADME (absorption, distribution, metabolism and excretion) measurements.

Information recorded for each patient includes age (years), gender, height (cm), family history of disease state or condition (yes/no), motivation rating (some/moderate/

great) and number and type of previous treatment regimens for the indicated disease or condition.

Volunteers taking part in this study are healthy adults (age 18 to 65 years) and roughly an equal number of males and females participate in the study. Volunteers with certain characteristics are equally distributed for placebo and stearoyl-CoA desaturase inhibitor treatment. In general, the volunteers treated with placebo have little or no response to treatment, whereas the volunteers treated with the stearoyl-CoA desaturase inhibitor show positive trends in their disease state or condition index at the conclusion of the study.